Br. J. Pharmac. (1970), **40**, 800–813.

Uptake of monoamines into central neurones and the blood-brain barrier in the infant rat

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Summary

1. A fluorescence histochemical study was undertaken to investigate the ability of systemically administered monoamines to penetrate into the central nervous system of infant rats.

2. It was found that after subcutaneous injections of large doses, L-noradrenaline, L-α-methyl-noradrenaline, DL-α-methyl-dopamine, L-α-methyl-dopa and 5-hydroxytryptamine could cross the blood-brain barrier of newborn to 2 week old rats and be taken up by neurones normally containing monoamine as well as by several neurones which normally contained no monoamine.

3. 5-Hydroxytryptamine containing neurones took up 5-hydroxytryptamine as well as catecholamines and α-methyl-dopa. The pinealocytes, which contain large amounts of 5-hydroxytryptamine, were depleted of their endogenous fluorescence by α-methyl-dopa and showed no selective uptake of catecholamines.

4. The uptake of monoamines was not antagonized by previous reserpine treatment or by inhibition of catecholamine synthesis, and occurred throughout the whole extent of the monoamine-containing neurones (cell body, axon, terminals).

5. The monoamines were also taken up by the endothelial cells and pericytes of capillaries in the central nervous system (CNS) which are thought to constitute the physical barrier to these substances in the adult.

6. The peripheral sympathetic nerves to pial blood vessels, the pineal and pituitary glands also took up the monoamines and α-methyl-dopa ; uptake was also noted in cells of the pituitary gland, in epithelial and connective tissue elements of the meninges and choroid plexuses and in the epithelial, connective and muscular tissue of pial blood vessels.

7. It is concluded that the blood-brain barrier to monoamines may not be fully developed in infant rats, at least for high levels of circulating monoamines ; the central monoamine-containing neurones possess the 'membrane pump' mechanism for uptake of monoamines from the time of birth, even though they are not fully developed morphologically.

Introduction

It is normally assumed that circulating monoamines (MA) do not enter the brain because of the existence of a blood-brain barrier, probably at the level of the brain blood vessels (Axelrod, Weil-Malherbe & Tomchick, 1959 ; Bertler, Falck & Rosen-

gren, 1963; Bertler, Falck, Owman & Rosengren, 1966; Fuxe, Hamberger & Malmfors, 1967; Fuxe & Hillarp, 1964; Fuxe & Ungerstedt, 1968a, b; Glowinski, Axelrod, Kopin & Wurtman, 1964; Glowinski, Kopin & Axelrod, 1965; Weil-Malherbe, Whitby & Axelrod, 1961). It has been shown that this barrier is partially due to the presence of monoamine oxidase (MAO) in the cytoplasm of endothelial cells and pericytes of capillaries of the central nervous system; there is probably a "physical" barrier as well, in that MA cannot easily penetrate the cell membrane of endothelial cells unless the MA is present in very high concentrations (Bertler *et al.*, 1963; Bertler *et al.*, 1966; Owman & Rosengren, 1967). The presence of L-dopa decarboxylase in addition to MAO in the capillary cells constitutes an enzymatic barrier to the amino-acid precursors of MA—L-3-4-dihydroxyphenylalanine (L-dopa) and L-5-hydroxytryptophan (5-HTP) (Bertler *et al.*, 1963; Bertler *et al.*, 1966; Owman & Rosengren, 1967). During the first few days of postnatal life the rat brain can take up small amounts of circulating catecholamines (CA), but on the whole the barrier is thought to be complete even in the newborn (Glowinski, Axelrod, Kopin & Wurtman, 1964).

The use of the fluorescence histochemical technique has provided evidence that in the adult, systemically administered MA can penetrate into those areas of the brain, and adjacent regions, which are known to lack a barrier to circulating substances, that is, the median eminence, the area postrema, the intercolumnar tubercle and the supraoptic crest (Bertler *et al.*, 1966; Fuxe *et al.*, 1967; Lichtensteiger & Langemann, 1966; Lichtensteiger, Mutzner & Langemann, 1967; Loizou, 1967). Experimentally induced lesions of the barrier result in permeation of the barrier by CA (Hamberger & Hamberger, 1966). After penetrating into the brain parenchyma the amines are taken up mainly by MA-containing neurones (Fuxe *et al.*, 1967; Lichtensteiger & Langemann, 1966; Lichtensteiger *et al.*, 1967; Loizou, 1967).

The object of the present studies was to investigate whether circulating MA could enter the brain parenchyma in regions other than those lacking the barrier in newborn and infant rats and be taken up by neurones. The amines studied were L-noradrenaline (NA), L-α-methyl-noradrenaline (α-methyl-NA), DL-α-methyl-dopamine (α-methyl-DA) and 5-hydroxytryptamine (5-HT); in addition the L-dopa derivative, L-α-methyl-dopa (which permeates the blood-brain barrier) was studied. Apart from brain and spinal cord, the pineal, pituitary and pial blood vessels were examined for comparative purposes.

Methods

Male and female rats of a laboratory inbred Wistar strain were used throughout this investigation (Table 1). The drugs used were reserpine (Serpasil, Ciba), nialamide (Niamid, Pfizer), DL-α-methyl-*p*-tyrosine methyl ester hydrochloride (H44/68, Hassle, Sweden), L-noradrenaline bitartrate (Koch-Light), L-α-methyl-noradrenaline (Corbasil HCl, Hoechst), DL-α-methyl-dopamine hydrobromide (H62/40, Hassle, Sweden), L-α-methyl-dopa (Aldomet, Merck), 5-hydroxytryptamine creatinine sulphate (L. Light), protriptyline hydrochloride (Merck), and imipramine hydrochloride (Tofranil, Geigy). Where appropriate the drugs were dissolved in 0·9% saline and injected subcutaneously, or in older animals intraperitoneally. Doses refer to the salt. The brain and spinal cord from these animals were examined with the fluorescence histochemical technique of Falck & Hillarp for the demonstration of biogenic amines (Falck & Owman, 1965). Details of the tech-

nique will be published elsewhere (Loizou, in preparation). With this technique the flurophore of CA and related substances emits a green to yellow-green light while that of 5-HT (or related substances) emits a yellow to orange-yellow light.

Three criteria were employed in order to decide whether an amine permeated the brain parenchyma or not:

(*a*) Appearance of specific fluorescence in walls of capillaries.

(*b*) Appearance of diffuse fluorescence in the parenchyma.

(*c*) Appearance of fluorescence in neurones induced by the exogenous substance following its uptake by the neurones; uptake of exogenous MA into normally fluorescing neurones was decided on the basis of either increased fluorescence intensity or shift in emitted colour of fluorescence. However, for more reliable observations, many animals were depleted of the endogenous MA using reserpine or H44/68, so that any observed fluorescence was attributed to the exogenous MA.

Results

α-Methyl-dopa

Qualitatively the results obtained in the 1 week old and older rats were similar.

With the higher doses given 6 h before death (e.g. 340 mg/kg in one 2 week old; 200 and 300 mg/kg in two 1 week old rats) or after a 1·5 h interval (e.g. 250 mg/kg in two adults) green fluorescence could be observed in the cells of brain capillaries and in the brain parenchyma. This clearly indicated passage of the amino-acid through the capillary wall. This amino-acid was selectively taken up by nearly all the CA-containing neurones (cell bodies, axons and terminals) and at higher doses or shorter intervals by neurones normally containing 5-HT also, as evidenced by an enhanced green fluorescence in all such neurones compared with the untreated controls (Dahlstrom & Fuxe, 1964; Fuxe, Hokfelt & Ungerstedt, 1969; Loizou, 1969). Outside the CNS there was uptake into nerve terminals or preterminal axons of the pial blood vessels. In the pineal gland the amino-acid caused a conspicuous to complete depletion of the yellow fluorescence (due to 5-HT) of the parenchyma, which consequently appeared dark or faintly yellow, and resulted in the appearance of strong green fluorescence (characteristic of CA) in the pineal nerves, which also normally have a strong yellow fluorescence due to 5-HT, taken up from the parenchyma. In the pituitary gland the amino-acid was taken up by CA-containing terminals of central and peripheral origin as well as by cells of the pars distalis.

In the newborn animals all the doses used resulted in a high to extremely high fluorescence in the brain parenchyma, choroid plexus, pineal, pituitary and pia mater so that no details of 'uptake' by individual structures could be observed. It was apparent that the amino-acid had penetrated into the brain parenchyma.

In order to assess the importance of intraneuronal storage mechanisms for the retention of α-methyl-dopa and/or its derivatives in CA-containing neurones, some 1 week old rats were pretreated with reserpine before α-methyl-dopa administration. In all the reserpine pretreated animals the retention of α-methyl-dopa (or its derivatives) was reduced (compared with the non-reserpinized rats); thus weak fluorescence was observed only in cell bodies of normally CA-containing neurones, in the

TABLE 1. *Experimental material studied*

Treatment		Age				
Monoamine or amino-acid	Other drugs	Newborn–3 days	1 week	2 weeks	3 weeks	Adult
α-Methyl-dopa, 100–340 mg/kg, s.c., 0·5–6·5 h	—	6	3	3	2	10
α-Methyl-dopa, 25 mg/kg, s.c., 0·5–1·5 h	—	3	–	–	–	–
α-Methyl-dopa, 100 mg/kg, s.c., 4–12·5 h	Reserpine 10 mg/kg, s.c., 10–30 h	–	8	–	–	–
α-Methyl-NA, 150–250 mg/kg, s.c., 0·5–1 h	—	3	2	2	2	–
α-Methyl-NA, 200–250 mg/kg, s.c., 0·5 –1 h	H44/68, 250 mg/kg, s.c., 5–9 h	3	2	–	–	–
α-Methyl-NA, 200 mg/kg, s.c., 0·33–1 h	H44/68, 250 mg/kg, s.c., 8·5 h + protriptyline or imipramine HCl, 50 mg/kg, s.c., 15–25 min before α-methyl-NA	6	–	–	–	–
α-Methyl-NA, 10–250 mg/kg, s.c., 0·5–1 h	Reserpine, 5–10 mg/kg, s.c., 4–6 h	13	2	2	–	–
α-Methyl-DA, 100–150 mg/kg, s.c., 0·5–1 h	—	2	2	2	–	–
α-Methyl-DA, 200–250 mg/kg, s.c., 0·5–1 h	H44/68, 250 mg/kg, s.c., 5–7 h	2	2	–	–	–
L-NA, 20–100 mg/kg, s.c., 0·33–1·5 h	Reserpine, 5–10 mg/kg, s.c., 4–7 h +nialamide, 200–500 mg/kg, s.c. or i.p., 0·5–3 h	5	5	4	–	3 (phenoxybenzamine hydrochloride was administered 10 min before L-NA)
5-HT, 300 mg/kg, s.c., 0·5–1 h	—	2	–	–	–	–
5-HT, 150–250 mg/kg, s.c., 0·25–1 h	Reserpine, 5–10 mg/kg, s.c., 3–5 h + nialamide, 50–250 mg/kg, s.c., 1–1·5 h	5	2	2	–	–

Numbers in columns indicate number of animals. Where appropriate, control animals were treated with reserpine, reserpine+nialamide or H44/68. Times in hours refer to the time of injection before death. Reserpine or H44/68 were administered so as to deplete the endogenous MA or CA content (respectively); nialamide was administered after reserpine, so as to inhibit MAO before administering 5-HT or L-NA.

five rats given reserpine 10–12 h + α-methyl-dopa 4, 5, 6 or 8 h before death. In the three rats given reserpine 27–30 h and α-methyl-dopa 10, 11 or 12 h before death, however, there was fluorescence in some terminals of the telencephalon normally storing DA, and stronger fluorescence in the cell bodies than in the other five reserpinized rats. No fluorescence developed in axons and terminals of the pial blood vessels and pineal gland in any of the above animals. Since reserpinized controls showed no fluorescence due to endogenous MA, the fluorescence seen in the reserpine + α-methyl-dopa treated rats was entirely due to α-methyl-dopa or its derivates.

α-Methyl-NA

This catecholamine was administered to normal newborn to 2 week old animals, and also to rats of the same age depleted of their endogenous CA with H44/68 or reserpine (Table 1). The results obtained were qualitatively similar in all animals. Uptake was seen in almost all the CA-containing nerve cell bodies of groups A1–A13 (Dahlstrom & Fuxe, 1964; Fuxe, Hokfelt & Ungerstedt, 1969; Loizou, 1969) as well as in many cell bodies in the area postrema, intercolumnar tubercle, spinal nucl. of the trigeminal nerve, in the cerebellar folia, near the aqueduct, in the premammillary nuclei, the periglomerular cells in the olfactory bulb and several 5-HT-containing cell bodies (see Figs. 1–4). There was uptake into CA-containing nerve fibre around the base of the medulla and descending in the spinal cord (Fig. 5) in ascending axons from the medulla-pons through mesencephalon to the forebrain, in axons in the medial forebrain bundle and stria terminalis (Fig. 6) and in

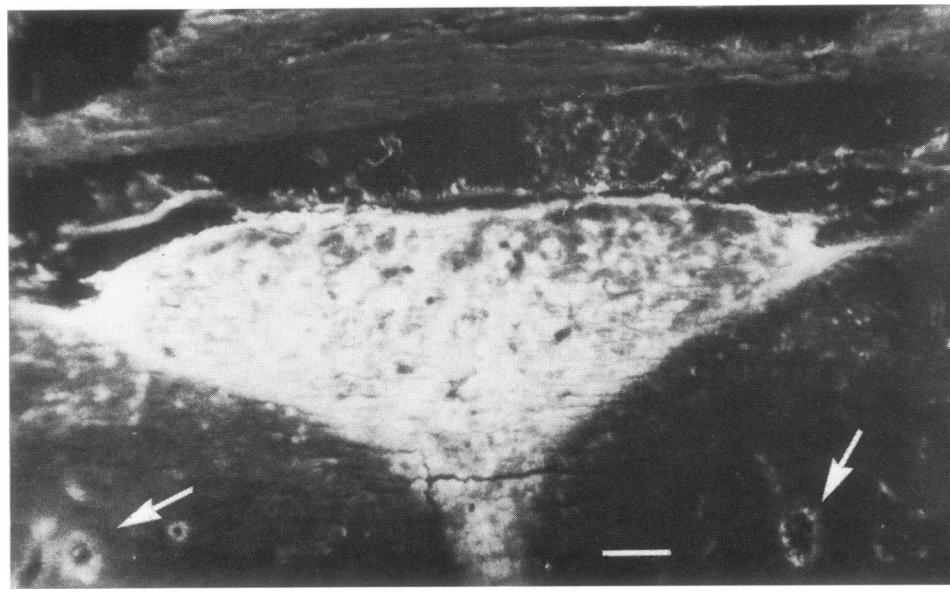

FIG. 1. Area postrema of newborn rat treated with H44/68 + protriptyline + α-methyl-NA. Note the very strong fluorescence in the area postrema (with some details of cellular uptake visible) and the fluorescence in the walls of blood vessels (arrows) in the dorsal part of the medulla; the strong fluorescence of the meninges overlying the area postrema as well as the fluorescence in the brain tissue is not visible due to photographic reduction of the intensity of fluorescence which was necessary to reveal detail in the area postrema. Transverse section. Calibration 64 μm.

others ascending through the tractus diagonalis to the cingulum or entering the olfactory bulb. CA-containing terminals also showed extensive uptake throughout the brain and spinal cord (Figs. 5 and 6), this being most pronounced in one week old animals.

In the regions outside the blood-brain barrier there was a strong to very strong yellow-green fluorescence partly diffuse, partly localized in cell bodies or nerve terminals (Fig. 1); such diffuse fluorescence was also seen in the spinal nucl. of the trigeminal nerve (Fig. 4) and in the glomeruli of the olfactory bulb. In these regions there was uptake of amine into cell bodies as well. The walls of brain capillaries (endothelial cells and pericytes) were mostly fluorescent and the brain parenchyma varied from dark to moderately strong green fluorescence. The larger blood vessels often contained fluorescent material in their lumina. There was uptake in the CA-containing fibres of pial blood vessels and of the pineal gland but not in the pineal parenchyma, which either retained its own yellow fluorescence or fluoresced yellow-green due to diffuse distribution of α-methyl-NA (particularly in the newborn rats,

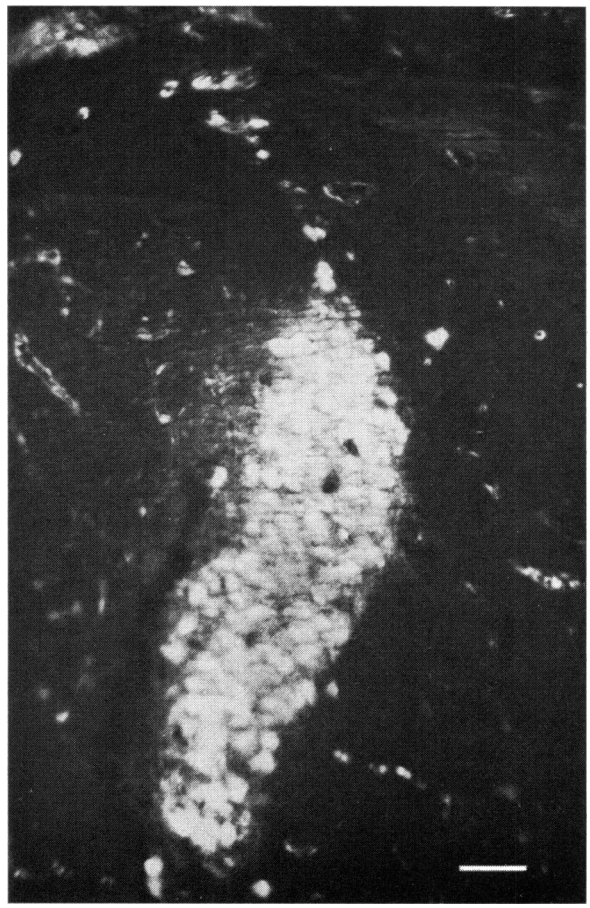

FIG. 2. Locus coeruleus (group A6 of Dahlstrom & Fuxe, 1964) of newborn rat treated with reserpine+α-methyl-NA. Note the strong fluorescence in the cell bodies entirely due to uptake of α-methyl-NA and the fluorescence in the walls of capillaries. Transverse section. Calibration 64 μm.

which lacked fluorescence in the parenchyma). There was uptake of the drug in the connective tissue and muscle coats of many pial blood vessels as well as in vessels forming the inner lining of the choroid plexuses, in the connective tissue of the pia-arachnoid matter, in nerve fibres in the neural lobe and pars intermedia and in cells of the pars distalis of the pituitary gland. In reserpine pretreated newborn rats it was found that the first signs of uptake of α-methyl-NA in cell bodies could be detected at a subcutaneous dose of 10–25 mg/kg after 3 to 4 hours. It was also found that the same amount of α-methyl-NA diluted in a larger volume of saline resulted in a much weaker and less extensive uptake into CNS neurones. No amine was found in the brain of 3 week old rats, except in the regions lacking the barrier. Characteristically no fluorescence could be detected in the walls of brain capillaries in these animals. The dose used (100 mg/kg) was toxic and the animals died within 10 min of injection. No such toxicity was seen in the younger rats.

In the newborn rats treated with H44/68 + protriptyline or imipramine + α-methyl-NA, neither protriptyline nor imipramine prevented the entry of α-methyl-NA into

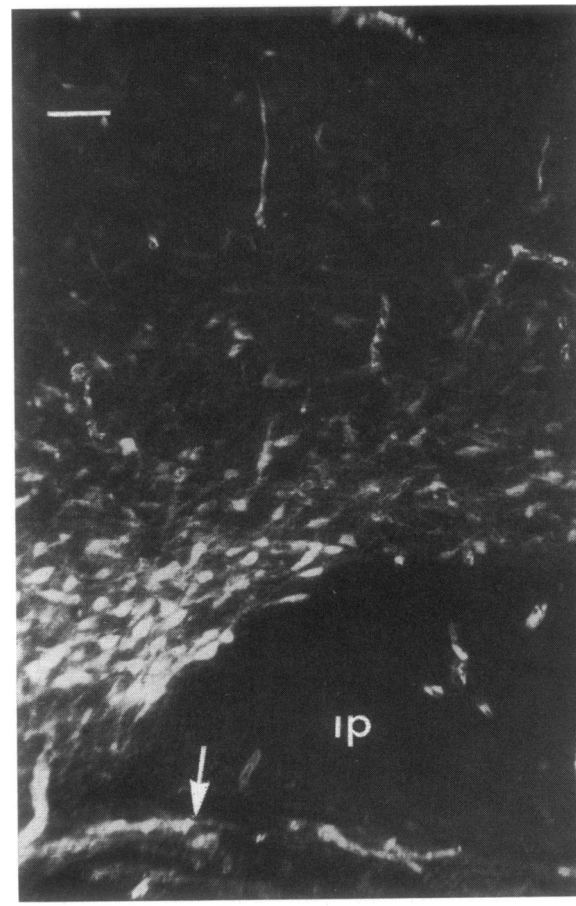

FIG. 3. Cell bodies of group A10 dorsal and lateral to interpeduncular nucleus (ip) of mesencephalon. 1 week old rat treated with H44/68+α-methyl-NA. Note the uptake into cell bodies (especially the more ventral ones) and in capillaries of the brain tissue and blood vessels in the pia matter (arrow). Transverse section. Calibration 64 μm.

the brain. Yet, protriptyline blocked the uptake into nerve cell bodies of groups A1–A7, into the presumed NA-containing terminals in the brain and spinal cord and into adrenergic fibres of the pineal and pial blood vessels, but it did not interfere with the uptake in other structures (for example into the DA-containing terminals of the telencephalon). Imipramine did not interfere with uptake in any structure.

α-Methyl-DA

This CA was administered to normal or H44/68 pretreated infant rats. There was no uptake in capillary cells or neurones in the 2 week old rats except in the areas outside the blood-brain barrier. However, this amine did enter the CNS of newborn and 1 week old rats and was taken up by neurones, capillary cells, pineal and pituitary in the same way as α-methyl-NA. One difference was noted in that α-methyl-DA was seen both in the internal blood vessel layer and the external epithelial layer of the choroid plexus.

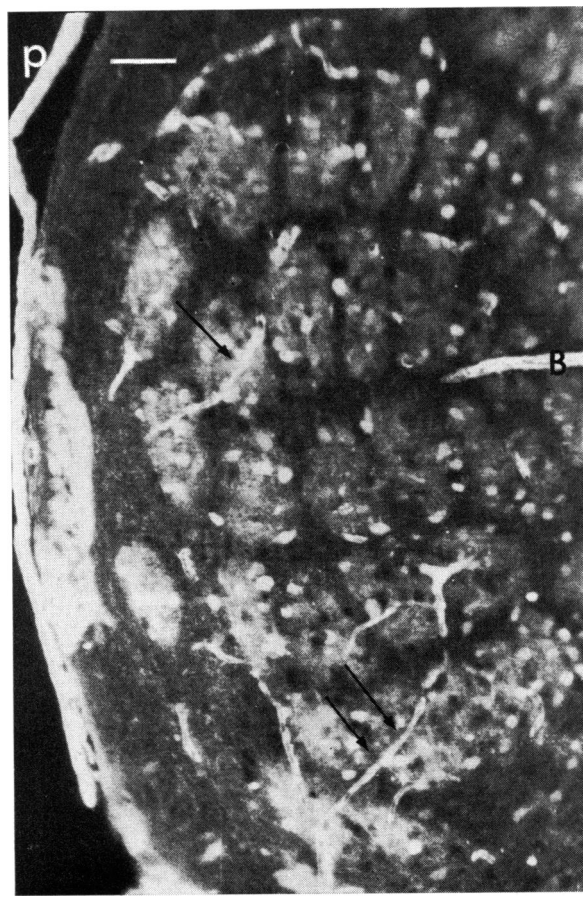

FIG. 4. Lateral part of caudal medulla oblongata of same rat as Fig. 3. Note the fluorescence in areas partly in the nucl. sensorius n.V, in small nerve cells, in the walls of capillaries (arrows) as well as in the lumen in one larger blood vessel (B) and in the pia mater (p). Transverse section. Calibration 64 μm.

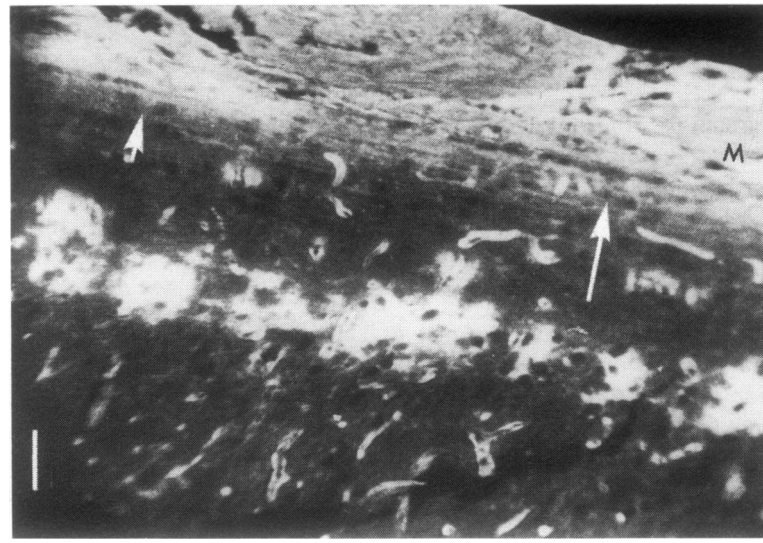

FIG. 5. Horizontal section through thoracic cord of 2 week old rat treated with α-methyl-NA. Note the presence of fluorescence in the walls of many capillaries, the uptake of α-methyl-NA in the MA-containing terminals of the lateral horn (the individual terminals are not distinguishable) and in fibres coursing along the edge of the cord (arrows). Strong fluorescence is also seen in the meninges (M). Calibration 64 μm.

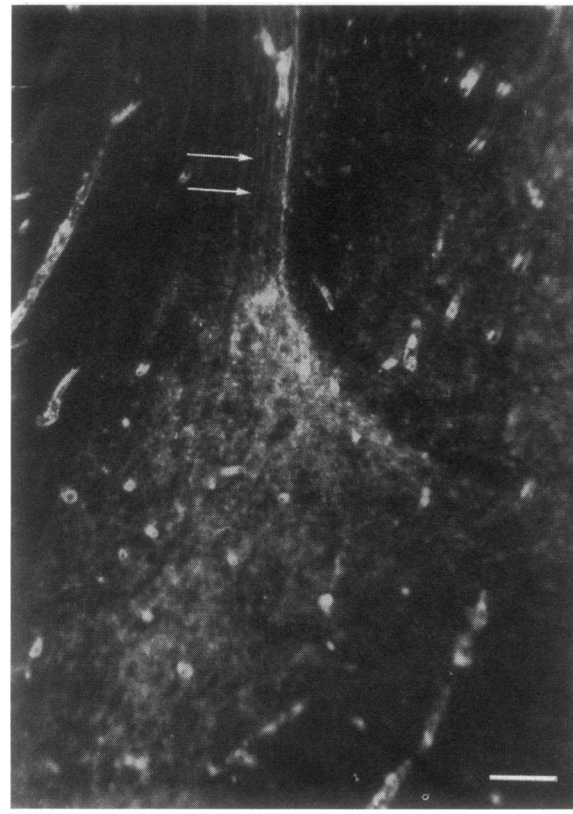

FIG. 6. Stria terminalis and central amygdaloid nucleus of 1 week old rat treated with H44/68+α-methyl-NA. Note the uptake into nerve fibres (arrows), in a dense plexus of very fine varicosities and in the walls and lumina of capillaries. Transverse section. Calibration 64 μm.

Noradrenaline

Noradrenaline was administered in animals pretreated with reserpine to deplete endogenous MA and nialamide to inhibit MAO. In 2 week old and adult rats the amine was only taken up by neurones and capillary cells located in areas lacking the blood-brain barrier. In the 2 day and 1 week old rats the amine entered the walls of capillaries and the parenchyma of the brain and spinal cord and was taken up by neurones as described for α-methyl-NA. Peripheral nerves, and the pituitary gland also took up NA. The pineal parenchyma was either diffusely yellow-green fluorescent (due to NA) or dark, but the pineal nerves were strongly green (or yellow-green) fluorescent, indicating selective uptake into these nerves (Fig. 7).

5-Hydroxytryptamine

This amine was administered to reserpine-nialamide pretreated rats and to two normal 2 day old rats. In the latter, weak diffuse 5-HT fluorescence was detectable

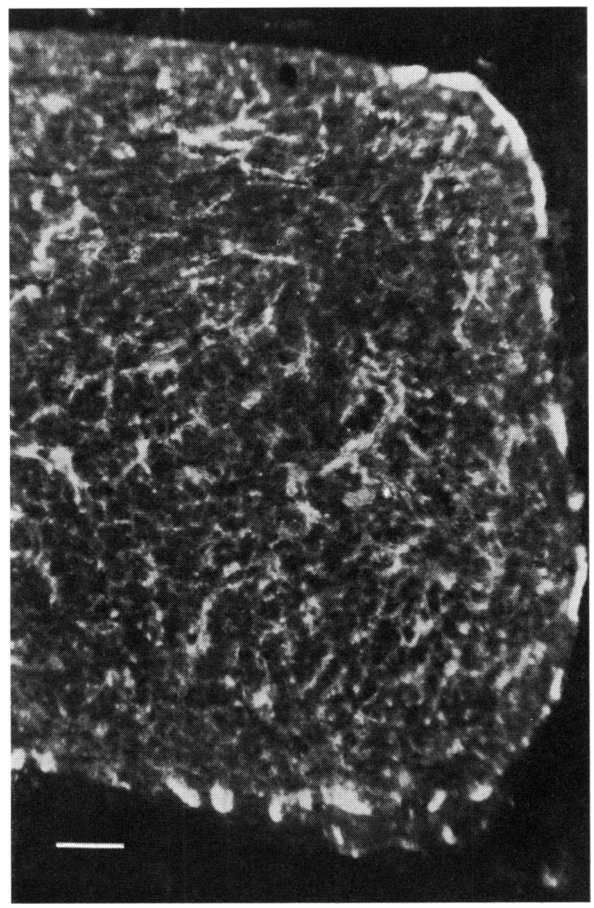

FIG. 7. Pineal gland of 1 week old rat treated with reserpine+nialamide+noradrenaline. Note the selective uptake of noradrenaline in the sympathetic nerve plexus in the pineal parenchyma and in nerve bundles in the capsule of the gland. Transverse section. Calibration 64 μm.

only in the regions outside the blood-brain barrier. In the pretreated animals there was no permeation into the brain at 2 weeks and it was only very slight at 3 and 7 days ; uptake was seen in a few 5-HT containing cell bodies, and in some cell bodies in the sensory spinal nucl. of the trigeminal nerve and in the cochlear nuclei. Some terminals in the lower brain stem also showed uptake (for example in the substantia gelatinosa) and so did descending axons from the medulla to the spinal cord. There was very strong fluorescence in the regions outside the blood-brain barrier, both diffuse and localized to cell bodies (for example in area postrema) or nerve terminals (in the median eminence, Fig. 8), in the pituitary gland, in nerve fibres and connective and muscular tissue of some pial blood vessels, in nerve fibres of the pineal gland and pineal parenchyma, in the pia mater, and the internal and external lining of the choroid plexuses.

Discussion

There are at least two routes via which the amines could have entered the CNS found in these experiments—either by direct permeation of the capillaries or by diffusion from the areas known to be outside the blood-brain barrier. In favour of the first alternative are the facts that fluorescence could be seen in the cytoplasm

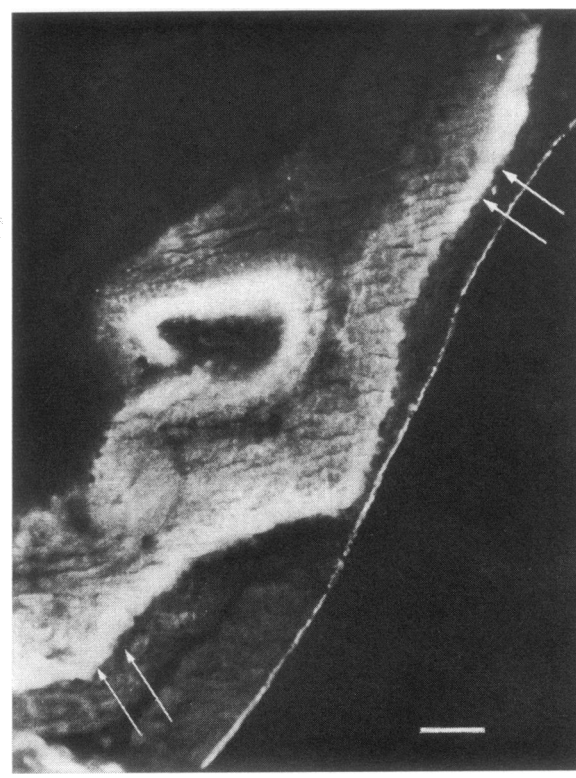

FIG. 8. Sagittal section through the median eminence and infundibular stem of the hypothalamus of 2 week old rat treated with reserpine+nialamide+5-HT. Note the accumulation of 5-HT in the external zone of the median eminence and infundibular stem (arrows). Calibration 64 μm.

of endothelial cells of brain capillaries, indicating that the amines did cross the inner surface of these cells, that fluorescence could often be seen in the brain parenchyma and in neuronal elements distant from any area lacking the blood-brain barrier or from the surface of the brain. It is also quite possible that enough amine could have reached the areas lacking the blood-brain barrier so that significant amounts could have diffused to adjacent tissue or have entered the cerebrospinal fluid of the ventricles and subarachnoid space, and circulated to the whole extent of the ventricular and subarachnoid system (Bulat & Supek, 1968a); the amine could then have diffused through the pia matter passively into the brain tissue and have been taken up by neurones located near the surface of the brain. However, one would expect that such a mechanism should be operative in all animals, but it was found that in the 2 week old and adult rats NA, α-methyl-DA and 5-HT did not enter the brain, although they reached the areas lacking the barrier in sufficiently high concentrations to diffuse either into the brain parenchyma or the cerebrospinal fluid. It is also noteworthy that after intraventricular injections of high doses of MA, these are taken up by neuronal elements only in a zone up to 300 μm wide surrounding the ventricles and the ventral part of the subarachnoid space (Fuxe & Ungerstedt, 1968a, b). It appears, therefore, that diffusion of MA from the ventricular and subarachnoid spaces cannot account for the widespread uptake seen in most animals studied.

On the whole the capillaries of the spinal cord appeared to be more permeable to CA than those in the brain. Of interest was the observation that certain areas of the brain—the spinal sensory nucl. of the trigeminal nerve—the cerebellum and the glomeruli of the olfactory bulbs were readily permeable to both CA and 5-HT, in infant animals. The permeability may also vary depending on the type of amine (see **Results**) and on the strain of rats, as suggested by the fact that in a different strain L-NA at a relatively low dose of 13 mg/kg subcutaneously penetrated the brain parenchyma of 2 week old rats to a considerable extent (Loizou, 1967).

The poor permeation of 5-HT into the brain was unexpected especially in view of the finding of Bulat & Supek (1968a, b) that this amine passes through the blood-brain barrier of adult rats.

It was directly demonstrated that MA were taken up by the entire MA-containing neurone (cell body, axon and terminal) right from the time of birth. The uptake was unaffected by prior depletion of endogenous MA stores by H44/68 or reserpine, indicating that the mechanism is independent of the functional capacity of storage granules (Lundborg, 1967) and that it most probably operates at the level of the nerve cell membrane, as already suggested by other workers (Fuxe & Ungerstedt, 1968a, b; Hamberger, 1967). It was of interest that in the preliminary experiments, imipramine did not block the uptake of α-methyl-NA but protriptyline did block this uptake in the presumed NA-containing neurones but not in those containing DA. These drugs are thought to be blockers of the membrane uptake mechanism of peripheral and central MA-containing neurones (Fuxe & Ungerstedt, 1968a, b; Hamberger, 1967).

The significance of uptake of MA by nerve cells that normally exhibit no fluorescence of their own is unknown. Similar findings have been reported by other workers (Fuxe & Ungerstedt, 1968a; Lichtensteiger, 1966). It was also noted that 5-HT-containing neurones took up catecholamines.

It was of interest that in 1 week old rats retention of α-methyl-dopa or its metabolites α-methyl-DA and α-methyl-NA (Henning, 1969; Uretsky & Seiden, 1969) was less extensive in reserpine pretreated than control rats; this suggests that this amino-acid or amines (which are resistant to MAO) are normally stored in storage granules as well as being able to accumulate in the cytoplasm of CA-containing neurones (Lundborg, 1967; Uretsky & Seiden, 1969). α-Methyl-dopa (or its metabolites) was taken up and retained in 5-HT containing neurones (at high doses, and/or short intervals, see **Results**) but it caused depletion of 5-HT fluorescence in pinealocytes; the latter effect may be due to its property of inhibiting 5-HTP-decarboxylase (Smith, 1960) which is probably the rate limiting enzyme step in the development of 5-HT stores in the pineal (Haganson, Des Gouttes & Owman, 1967).

It can be concluded that the blood-brain barrier of the newborn rat to monoamines is not as developed as that of the adult at least for high levels of circulating MA and that the MA-containing neurones possess a membrane localized uptake mechanism ('membrane pump') for MA right from the time of birth even though they are not fully developed morphologically (Loizou, in preparation).

This work was supported by a research scholarship from the Wellcome Trust Foundation. The author is grateful to Professor J. H. Wolstencroft for supervising the work and reading the manuscript and to Dr. L. L. Iversen for reading and commenting on the manuscript.

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(*Received July* 10, 1970)